A STUDY BY NITROGEN-15 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF THE STATE OF HISTIDINE IN THE CATALYTIC TRIAD OF α-LYTIC PROTEASE[1]

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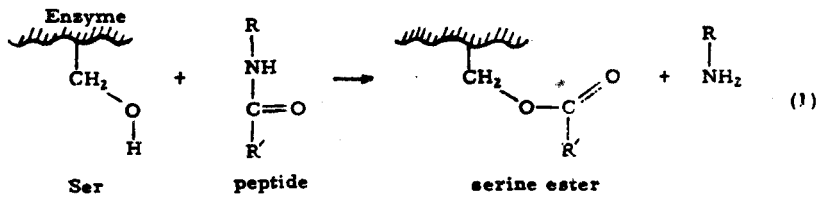
Hydrolytic cleavage of peptide bonds is an energetically favorable reaction, but one that normally takes place very slowly at room temperature, even in the presence of rather strong acids or bases. It can be strongly catalyzed by many proteases, and much effort has been expended to determine how these have the ability to increase the rate of hydrolysis by a million-fold or more in neutral solutions. One of the types of proteases, the serine-protease family, is characterized by the presence at the active site of a "catalytic triad" comprised of the side-chain residues of serine, histidine and aspartic acid.

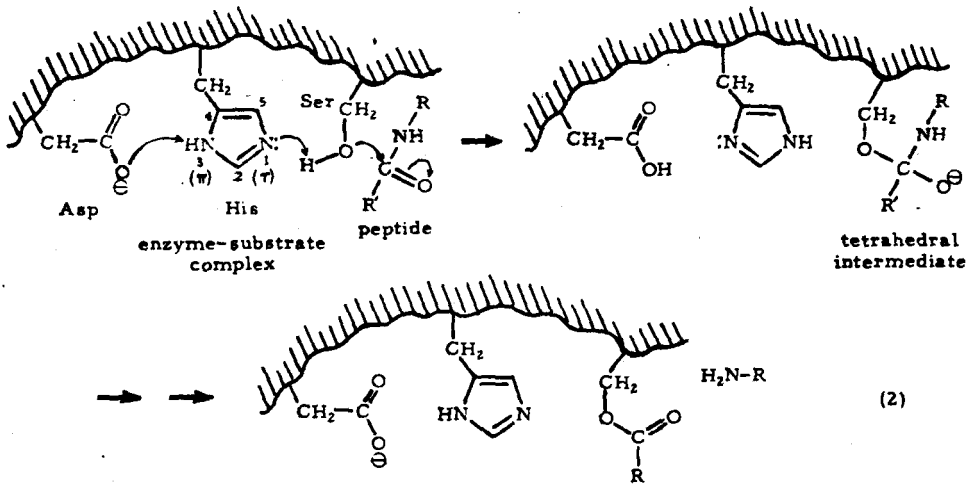
A key feature of the mechanism of action of the serine proteases is an attack by the hydroxyl group of the serine located at the active site on the carbonyl group of the peptide at the cleavage point, forming an ester and liberating an amine group, Eqn 1.[2]



Because uncatalyzed alcoholysis of peptide bonds, like hydrolysis of such bonds, is thermodynamically favorable, but kinetically very slow, the fact that the ester is formed is not itself helpful in understanding why the enzymatic hydrolysis is so facile.

The vital role of histidine in catalysis by serine protease is well established,[2] and Blow's discovery[3] for α-chymotrypsin that Asp 102, His 57 and Ser 195 seem aligned to act in some concerted fashion in peptide hydrolysis led to the proposal of a "charge-relay" mechanism which was later modified by Richards and coworkers[4] to a sequence wherein attack of the serine hydroxyl oxygen on peptide complexed with the enzyme occurs simultaneously with removal of the serine hydroxyl hydrogen by histidine at N1 (the τ nitrogen) and

transfer of a proton from N3 (the π nitrogen) to the carboxylate oxygen of the aspartyl residue (Eqn 2).



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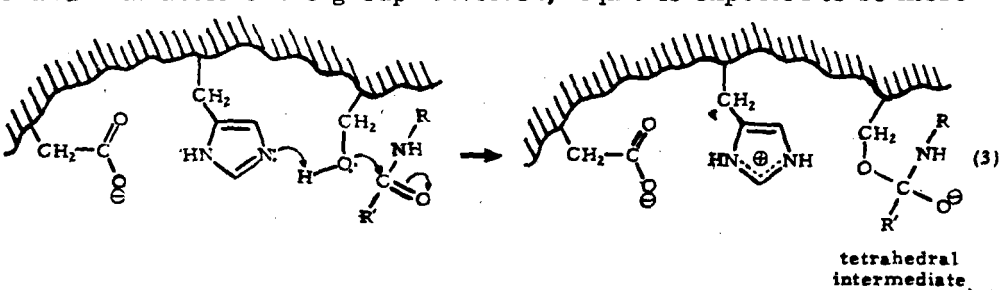
The result of this process is formation of the so-called "tetrahedral intermediate" which decomposes to the serine ester with cleavage of the peptide bond.

If the charge-relay mechanism is to account for the catalytic activity of the enzyme, it is necessary that it be energetically reasonable, at least to the degree that the postulated products need to be more stable than those that would be formed without transfer of a proton to the aspartyl carboxylate anion.[5] Unless being incorporated into an enzyme drastically changes the chemical character of the groups involved, Eqn 3 is expected to be more



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favorable than the first step of Eqn 2 by somewhat more than 3 kcal/mole, because imidazolium cations ($pK_a \sim 7$) are normally weaker acids than the terminal carboxyl of aspartic acid ($pK_a \sim 4.5$).

This difficulty with the charge-relay mechanism has been addressed by Richards and coworkers[14] who prepared α-lytic protease (a member of the serine-protease family) enriched in ^{13}C at the 2-position of its single histidine. They reported that the NMR spectrum of the enriched enzyme indicated that the histidine does not become protonated until the pH is reduced to below 4. The

corollary of this is that the free aspartyl carboxyl, located in a hydrophobic pocket of the enzyme, is the group having the pK_a of 6.7, and that it produces inactive enzyme when protonated. The Richards work suggests that the structure of the enzyme in the neighborhood of the catalytic triad is such as to make the histidine imidazole ring a weaker base by three orders of magnitude and the aspartyl carboxyl a weaker acid by two orders of magnitude. These results favor formation of the tetrahedral intermediate by the route of Eqn 2 rather than Eqn 3.

We have studied the ionization behavior of the histidine of the catalytic triad of α-lytic protease using ^{15}N NMR spectroscopy. This technique is expected to be especially informative about the state of protonation, hydrogen-bond formation, and tautomeric equilibrium of imidazole rings on the basis of the nitrogen NMR studies of Rüterjans and coworkers[6] with ^{15}N-enriched histidine, and ^{14}N results obtained by Witanowski and coworkers[7] on imidazoles. It seemed especially appropriate to investigate α-lytic protease because of its similarity to the mammalian serine proteases and the extensive studies already made of its ionization behavior. To achieve efficient and specific incorporation of ^{15}N-labeled histidine into α-lytic protease, we have induced and isolated an auxotroph of myxobacter 495 for which histidine is an essential amino acid. With the aid of this mutant, it has been possible to obtain substantial quantities of ^{15}N-enriched α-lytic protease with relatively small amounts of labeled histidine in the growth medium.

RESULTS

A representative ^{15}N NMR spectrum of α-lytic protease obtained from 3-(π)-^{15}N-labeled histidine is shown in Fig. 1. Other than the broad, relatively weak

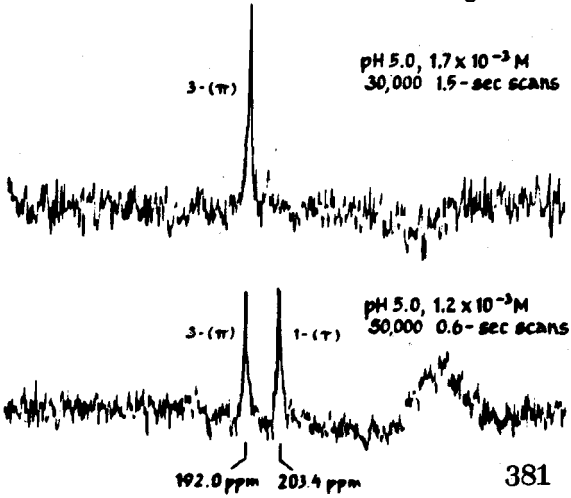


Figure 1. Proton-coupled ^{15}N NMR spectra, at 18.2 MHz, of α-lytic protease from auxotroph of myxobacter 495 + ^{15}N-labeled histidine (1 His/molecule, MW 19,860).

resonance at 245–265 ppm arising from the amide nitrogens in the peptide backbone of the enzyme, only a single resonance is observed, which can be assigned to the 3-(π)-nitrogen of the histidine of the catalytic triad. The pH dependence of the position of this resonance is shown in Fig. 2, and it will be seen that there is an upfield chemical shift with increasing pH from 191.6 ppm at pH 4.5 to 199.4 ppm at pH 8.5. The shape of the pH curve is consistent with titration of an acid with a pK_a of 7.0.

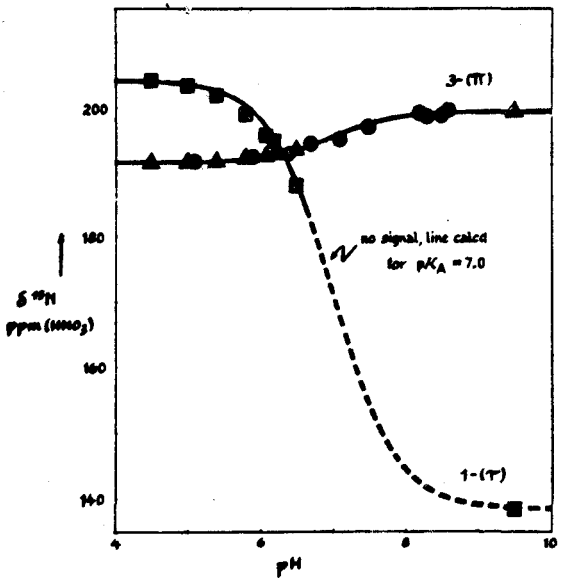


Figure 2.
Dependence of ^{15}N shifts of ^{15}N-enriched histidine nitrogens in α-lytic protease as a function of pH.
●, ^{15}N-enriched at N3;
▲, ■, enriched at N1 and N3, respectively.

The nitrogen NMR spectrum of the α-lytic protease prepared from doubly labeled histidine shows two ^{15}N resonances (Fig. 1). One of these behaves identically with variation of pH as for the singly labeled enzyme, and this is clearly the 3-(π)-nitrogen of the imidazole ring (Fig. 2).

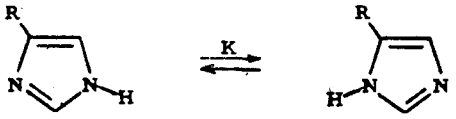
In contrast to the rather small (7.8 ppm) upfield chemical-shift change observed for N3 on going from pH 4.5 to 8.3, N1 (τ) exhibits a large (66 ppm) downfield shift change in going from 204.2 ppm at pH 4.5 to 138 ppm at pH 9.5. Again, the histidine residue is clearly acting as an acid with a pK_a of about 7. The positions of these resonances are fully reversible over the pH range investigated.

A serious problem was encountered with the N1 resonance over the pH range 6.5 – 9.0 when the signal strength simply decreased below limits of detection. Paramagnetic ions are especially likely to be the source of the loss of signal intensity at the intermediate pH values for the following reasons: 1) although many precautions were taken to eliminate contamination by heavy-metal ions, atomic absorption analyses of an aliquot of the solution used for

the NMR spectra indicated the presence of 1.9 ppm of iron and 0.17 ppm of copper; 2) decrease in the line width of the ^{15}N resonance at higher pH values is expected because either the 0.3 mM EDTA added to these solutions or hydroxide ions compete for binding of the metal ions; 3) the N3 resonance of 1-methylimidazole is very greatly broadened to near the limit of detectability at pH 6.0 in the presence of 10^{-5} M copper ions.[8]

In any case, the ^{15}N chemical shifts that could be measured for N1 (τ) over the range of pH 4.5 and 9.5 serve at least as boundary values for estimating the pK_a of histidine of α-lytic protease. Thus, $pK_a = 7.0$, which fits the pH dependence of the resonance of N3 in the enzyme, gives an excellent fit to the available data for N1 (τ) as well (Fig. 2). We conclude therefore that the histidine of α-lytic protease behaves as a normal imidazole with respect to basicity. We turn next to the problem of using the NMR data to extract as much structural information as possible about the condition of the histidine of α-lytic protease at different pH values.

A characteristic of imidazoles with an N-H bond is that they exist as a rapidly equilibrating mixture of tautomeric forms, Eqn 4. These equilibria



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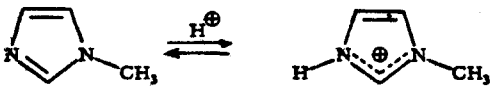
are established in water solution at room temperature, with rate constants >500 sec^{-1}. The equilibrium constants K of Eqn 4 depend on the nature of R but obviously, for imidazole itself, K will be unity, and because equilibration is fast, the nitrogen NMR resonances appear to have the same chemical shift which, for imidazole, is 171 ppm (Table 1).

However, it is well known from a variety of studies that the >NH and >N nitrogens of imidazole should have a very large difference in chemical shift.[7] 1-Methylimidazole 1a provides an excellent model to demonstrate this, because equilibration of the type represented by Eqn 4 does not occur at a measurable rate in water at room temperature.



1a 1b (5)

Table I. ^{15}N Chemical shifts of some imidazole derivatives in acid and neutral aqueous solutions at 25° C in ppm. relative to $D^{15}NO_3$[a]

Compound		Imidazolium cation[b]	Imidazole[c]	Δ[d]	pK_a	Ave. shift of imidazolium cation[e]	Ave. shift of imidazole[f]
Imidazole		202.0	171.0	31.0	6.95	202.0	171.0
1-Methylimidazole	N1	204.1	211.5	-7.4		203.9	169.9
	N3	203.6	128.5	75.1			
4-Methylimidazole	N1	202.6	172.8	29.8	7.5	200.6	168.6
	N3	198.6	164.4	34.2			
Imidazole-4-acetic acid	N1	203.0	180.0	23.0	7.35	201.4	167.8
	N3	199.7	155.6	44.1			
Histidine[g]	N1	201.5	196.5	5.0	6.2	200.3	169.8
	N3	199.0	143.1	55.9			
	N1		180.8	20.7			168.9
	N3		157.0	42.0			
α-Lytic protease	N1	204.2	138.0	66.2	7.0	197.9	168.7
	N3	191.6	199.4	-7.8			

[a]Positive shifts are upfield. [b]Shifts under conditions of full protonation. [c]Shifts under conditions of no protonation. [d]Changes in shift from cationic to neutral imidazole ring. [e]Average shift of nitrogens in imidazolium cation. [f]Average shift of nitrogens in neutral imidazole ring. [g]Shift data from Rüterjans and coworkers[6] recalculated to $D^{15}NO_3$ standard. One set of data for neutral imidazole is for histidine amphion and the other for the histidine anion.

The difference in ^{15}N shift between the nitrogens of 1a is 83.3 ppm, and we expect that there would be nearly the same difference in shift between the nitrogens of imidazole if equilibration by Eqn 4 were slow. That 1a is a reasonable model for the shifts of the individual tautomers is supported by the fact that the average of the shifts of 1a, 169.9 ppm, is only 1.1 ppm different from the observed average shift of imidazole itself.

On protonation of imidazole, the average position of the nitrogen resonances moves upfield by 31 ppm. The nitrogens are equivalent in the imidazolium cation 2 so the problem posed by the tautomeric equilibrium of Eqn 4 no

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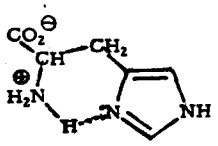
longer applies. Again, 1-methylimidazole seems to behave like imidazole in that the nitrogen shifts of its conjugate acid 1b, formed as in Eqn 5, differ by only 0.5 ppm and their average is less than 2 ppm different from the nitrogen shifts of 2.

The data of Table 1 show that the influence of a saturated 4-substituent on the ^{15}N shifts of imidazoles represent at most a rather small perturbation on the system. Thus, other than for α-lytic protease, the average shifts of the nitrogens of the 4-substituted imidazolium cations differ from those of imidazole or 1-methylimidazole by less than 4 ppm, and the average shifts of the corresponding neutral forms differ from those of their counterparts by less than 3.3 ppm.

The magnitude of the K of Eqn 4 is of special interest for α-lytic protease because it could give information about the surroundings of the histidyl residue of the enzyme. Histidine itself is not a good model for what one should expect for a non-terminal histidyl residue in a peptide chain because K for histidine is greatly affected by hydrogen-bonding interactions of N3 (π) of the imidazole ring with the α-ammonium group.[6]

When the imidazole ring of the histidine in α-lytic protease is not protonated, it has what appears to be quite normal, average nitrogen shifts (Table 1). However, K of Eqn 4 is clearly greater than 1, because the N3 (π) shift is upfield of the N1 (τ) shift in contrast to free histidine where the opposite is true.[6] Furthermore, K is less than 1 for 4-methylimidazole,[9] and this indicates that, for α-lytic protease, there is some interaction perturbing the tautomeric equilibrium in a manner analogous to (but opposite in direction to)

the way that the ammonium group of free histidine interacts with the N3 nitrogen (5) at pH 8 to cause K to be very much less than 1.[6] Such an interaction is possible with the carboxylate anion of the Asp residue making up the



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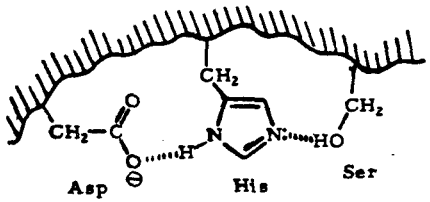

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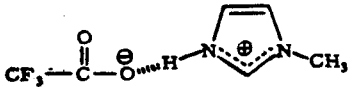
catalytic triad 4 and may be reinforced by further hydrogen bonding of N1 (τ) to serine. Judging from the shifts of 1-methylimidazole, K appears to be about 6 for the imidazole ring in α-lytic protease, which corresponds to stabilization by the interactions represented by 4 of about 1.1 kcal/mole. It is significant that other studies of histidyl residues in peptides,[10] thyrotropin-releasing factor[11] and myoglobins[12] show that $K < 1$ for these substances.

At low pH, the average ^{15}N shifts of the imidazolium cation of α-lytic protease are about 2.8 ppm more toward lower field than for the cations of the other 4-substituted imidazoles studied. Furthermore, the chemical-shift difference between N1 and N3 is about 12.6 ppm compared to an average of 3.5 ppm for the other 4-substituted imidazolium cations. This behavior has a close parallel to that observed for 1-methylimidazole in chloroform solution in the presence of an equivalent of trifluoroacetic acid. In this solution, the average ^{15}N shift is 6 ppm less than that of the protonated form in water, and the difference between the N1 and N3 shifts is 16 ppm.[9] The fact that the average shifts are less than normal suggests that there is some (probably small) degree of charge transfer from the imidazolium cations to a neighboring carboxylate anion as the result of hydrogen bonding, 5 and 6. The electrical asymmetry produced by the hydrogen bond then accounts for the differences in ^{15}N shifts between N1 and N3.



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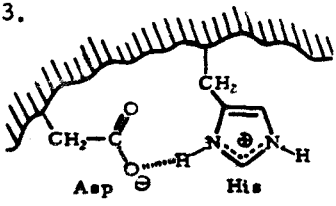

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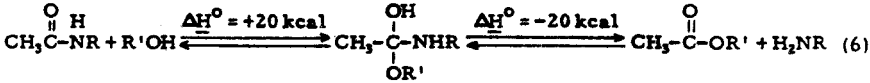
Trifluoroacetic acid in chloroform may not be the equivalent of the free carboxyl of an aspartyl residue in a hydrophobic region of an enzyme, but there can be no question that the pattern of shifts is very similar.

DISCUSSION

If the charge-relay mechanism for serine proteases requires that the pK_a of the Asp residue in the catalytic triad be comparable to or larger than the pK_a of the conjugate acid of the histidine of α-lytic protease, then the changes in the ^{15}N chemical shifts of this histidine with pH are most simply regarded as eliminating this mechanism as an important element in explaining why the catalytic triad is so effective.

What then is the role of the His-Asp couple of the triad? From published thermochemical data, bond energies, and the mean of about 5 kcal derived by Pople and coworkers[13] for the stabilization energy of the structural unit $-O-\overset{|}{C}-O-$, one can estimate that addition of ethanol to *N*-butylacetamide in the gas phase to form a tetrahedral intermediate 7 has ΔH° ~20 kcal, and decomposition to ethyl acetate plus butanamine has ΔH° ~ −20 kcal, Eqn 6 (R = $-C_4H_9$, R' = $-C_2H_5$)



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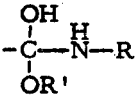
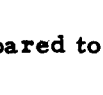
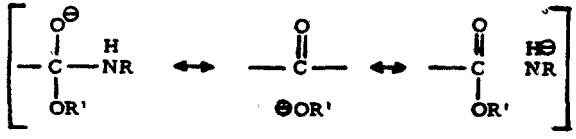
Neglecting possible differences in solvating energies of the participants, the extent to which ΔH° for formation of 7 would become more favorable in solution with alkoxide ion in place of an alcohol depends on the difference in acidity between 7 and the alcohol. This difference is difficult to evaluate, because little information is available to decide on how great an increase in acidity one should expect for the OH of the structural entity



as compared to



However, we do know that methoxyacetic acid is a twenty-times stronger acid than acetic acid, and the alkoxy group in this acid is one bond farther away from the acidic proton than in 7. Furthermore, there is no possibility of hyperconjugative stabilization of the methoxyacetate anion by resonance interactions of the type exemplified by 8.



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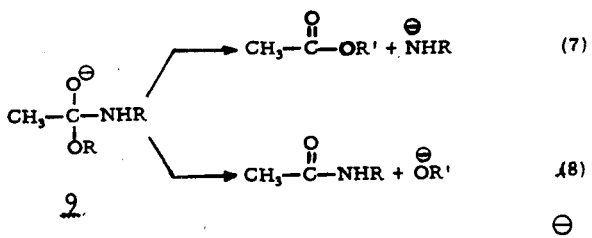
An intriguing possibility suggested by Pople's theoretical calculations[13] is that the stereochemistry of the tetrahedral intermediate is fixed by the structure of the enzyme to take full advantage of the very large potential stabilization (>11 kcal) of $RO-\underset{|}{\overset{|}{C}}-O^{\ominus}$ if the rotational angle around its RO-C bond were to have the optimum value.

Cleavage of 7 to amine plus ester and reversion to amide plus alcohol (Eqn 6) were estimated above to have essentially equal ΔH values. However, the decomposition reactions of the conjugate base of 7 (9) to amide plus alkoxide or ester plus amide anions are surely not equally favorable (Eqns 7 and 8).



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Indeed, the difference in pK_a between amines ionizing to $R-\overset{\ominus}{N}H$ and alcohols to $R\overset{\ominus}{O}$ is $\sim 10^{15}$, which corresponds to 20 kcal and suggests that Eqn 7 may not even be energetically favorable. Consequently, a powerful acid catalysis is expected for decomposition of 9 to ester and amine. Here, the imidazolium cation of the protonated His-Asp couple should be highly effective, and an important function of the local environment in the enzyme-substrate complex may be to prevent this cation from becoming deprotonated.

There are three reasonable roles of the aspartyl carboxylate group in this series of steps. One would be to favor the proper imidazole tautomer, another to orient the imidazole ring throughout the reaction to best advantage, and the third, to offer some assistance to stabilizing the imidazolium cation. That there may be some degree of stabilization arising from the imidazolium-carboxylate interaction in α-lytic protease corresponding to 6 seems possible from the fact that the pK_a of the histidyl residue in this enzyme is 7.0, even though many histidyl residues in other proteins have pK_a values less than 6.[14]

To summarize, the histidine of the catalytic triad of α-lytic protease appears to have a base strength which is essentially normal for an imidazole derivative but, in the pH range where the enzymatic activity is high, the histidine tautomer is favored with the hydrogen located on N3 (π), as the result of hydrogen bonding to the asparate anion and possibly the serine hydroxyl. Thus, the ^{15}N NMR shifts support the general geometry postulated for the charge-

relay mechanism but not the idea of an unusually weakly basic histidine or an unusually strongly basic asparate carboxylate anion.

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